AN AUTORADIOGRAPHIC STUDY OF THE RELATION BETWEEN HEMOCYTES AND CONNECTIVE TISSUE IN THE WAX MOTH, GALLERIA MELLONELLA L.[1]

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Considerable uncertainty exists as to the origin of the connective tissue sheaths around insect organs and the possible participation of blood cells in the development of connective tissue. Lazarenko (1925) made the first significant study of connective tissue in insects, and suggested that hemocytes participate in its formation. Wermel (1938) agreed with this, and so did Wigglesworth (1956) though the latter author considered the connective tissue sheath around the central nervous system, the neural lamella, a special case. Wigglesworth thought that the neural lamella of *Rhodnius* was secreted by an underlying layer of cells, the sheath cells, but that hemocytes probably contributed mucopolysaccharide to it. In contrast, Scharrer (1939) thought that the neural lamella of cockroaches was secreted entirely by the underlying sheath cells, and Bonhag and Arnold (1961) thought likewise for the connective tissue sheath around ovarioles. In moth pupae the larval connective tissue sheaths are destroyed, the organs then reorganized into their adult form, and then a new connective tissue sheath is formed. Ashhurst and Richards (1964a) concluded that one type of hemocyte (the adipohemocytes) participates in destruction of the larval neural lamella in moth pupae, but that hemocytes play no role in the later formation of the adult neural lamella around the nerve cord. All of the above studies were based on subjective deductions from histological appearances. The present study represents an attempt to obtain unambiguous objective data for the neural lamella in moth pupae, using a radioautographic technique. Support was obtained for the conclusions of Ashhurst and Richards.

MATERIALS AND METHODS

Larvae of the wax moth, *Galleria mellonella* L., were reared on an artificial diet (Haydak, 1940) at 39° C. and 75% R.H. in darkness.

Hemocyte counts (HC) were made by chilling animals in a refrigerator to deter blood coagulation, removing 2 μl. of blood, diluting it 70 × in a quick-diluting micropipette which could be twirled for mixing (Fig. 1), and then counting in a standard Levy hemocytometer. To obtain blood, incisions were made on the mid-dorsal line of larvae, in the lateral thoracic wall of pupae. The 2-μl. sample

was taken from the third drop of blood that exuded. The stated values are averages of determinations from nine different individuals.

Hemocytes were labeled with tritiated thymidine obtained from Schwarz Bio-Research Inc. The samples used had specific activities of 1.9 c./mM = 0.50 mc./ml. Injections of 5 μl. (= 2.5 μc.) were used with larvae and pupae of approximately 200 mg. live weight. In some cases, two or even three injections were made at successive intervals to increase the percentage of labeled cells.
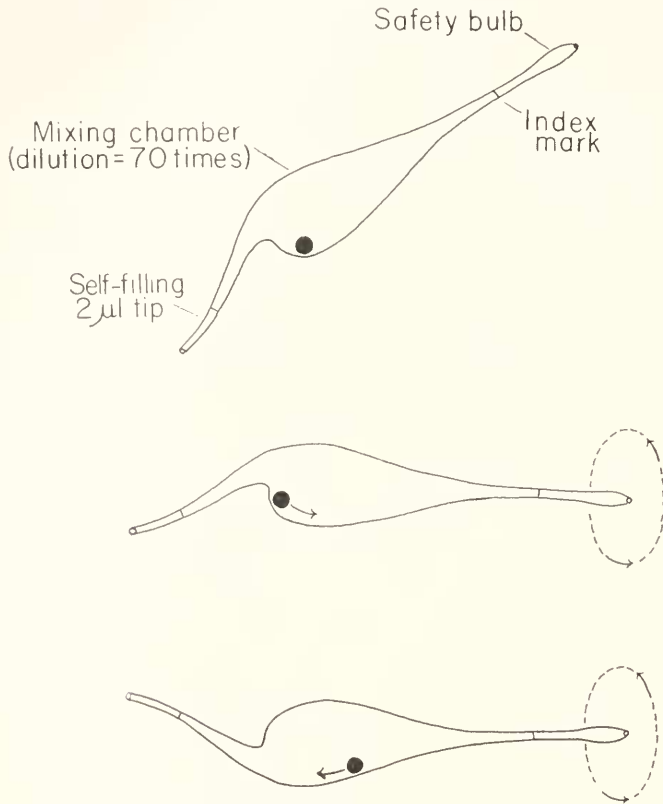

FIGURE 1. Diagrams showing the quick-diluting micropipette (2 μl.) and method of using it.

Blood transfusions from labeled prepupae and pupae to other prepupae and pupae of the same age were made in a cool room (15° C.) six hours after injection of H^3 thymidine into the donor. The receiving prepupae and pupae were bled as much as feasible (40–60 μl.) and then given a corresponding amount of blood from the labeled donor. The transfused animals were returned to a 35° cabinet to continue development.

Parabiotic twins were made using a labeled and a normal pupa of the same age. A few crystals of phenylthiourea, streptomycin sulfate and K penicillin were placed around the wound site before sealing the partners together with beeswax. After the operation, the pupae were pumped alternately with finger pressure to

mix the two blood streams and to assure free flow of blood with hemocytes from the donor to the pupa receiving labeled hemocytes.

The fate of labeled hemocytes was checked by examination of serial sections. At intervals of 18, 72 and 92 hours after pupation, pupae were killed by injection of Zenker's fluid into the thorax. The insects were then opened and fixed for 18–20 hours, washed overnight, dehydrated through an ethanol series, embedded in Tropical Ester Wax, and sectioned. Alternate sections from the ribbon were mounted on two sets of slides. One set was stained with Mallory's triple stain, the other set was used for autoradiography.

Autoradiographs were prepared by the method of Joftes (1959), using Nuclear Emulsion type NTB3 and exposing for 2–3 weeks. Unincorporated label, RNA, etc. were removed prior to autoradiography by treating the sections with 10% perchloric acid for two hours at 20° (Woods and Taylor, 1959).

RESULTS

Prior to using autoradiography to study the role of hemocytes in connective tissue formation it is necessary to obtain labeled hemocytes. Using tritiated thymidine it is possible to label nuclei permanently but only during mitosis. As a prelude, then, it is necessary to know when mitoses occur in hemocytes, to verify that mitoses can be stimulated to occur, and to learn something of the life cycle and life expectancy of hemocytes.

Normal hemocyte counts and the effects of bleeding

Stephens (1963) has recorded the mean hemocyte concentration or hemocyte count per mm.^{3} of blood (HC) in normal larvae of *G. mellonella* as 33,200 ± 1200. In insects in general the HC can change with age and stage due to an actual change in total hemocyte number, or the amount of plasma, or the number of cells adhering to the surfaces of various organs (Jones, 1962; Wheeler, 1963).

Our values for HC in *G. mellonella* were considerably lower than those given by Stephens but this may be due to the determinations having been made at a later time in development and utilizing the third drop of blood to exude from chilled individuals. At the end of larval development, when the larva has finished spinning its cocoon, the HC was found to average 15,000/mm^3. The value fell to 4000 in the motionless pharate pupa which is about to shed the larval skin, then rose gradually to regain the 15,000/mm.^{3} value about 12 hours later. The value then again decreased to about half this after another 12 hours (Figs. 2–3). How much of these changes may be due to each of the possible factors listed in the preceding paragraph was not determined; the values stated here represent only hemocyte concentrations in the circulating blood of chilled specimens.

Many years ago Dakhnoff (1938) reported that bleeding a larva of *G. mellonella* resulted in the production of excessive numbers of mitoses. Since it is only at mitosis that H^3 thymidine is incorporated as a permanent label in nuclei, the report suggests a method for obtaining a reasonable percentage of labeled hemocytes for use in transfusions and parabiotic twins. But we needed not only to verify Dakhnoff's report but also to see if the same increase in hemocyte count occurred during prepupal and early pupal stages. For this purpose late larvae

and early pupae were bled either a small drop (*ca.* 1 μl.) or a large drop (*ca.* 5 μl.), and the HC determined after various intervals of time in comparison to HC values of normal individuals. As is shown by the curves in Figures 2 and 3, the bleeding resulted in an approximate doubling of the HC in the hemolymph. The time required for this doubling was longer in the pupa than in the late larva but both showed the phenomenon. And in both there was a stimulation of mitotic activity.

Bleeding did cause a delay in the pupation of larvae and in the development of pupae but this, being known, does not interfere with the tests to be reported in subsequent parts of this paper.
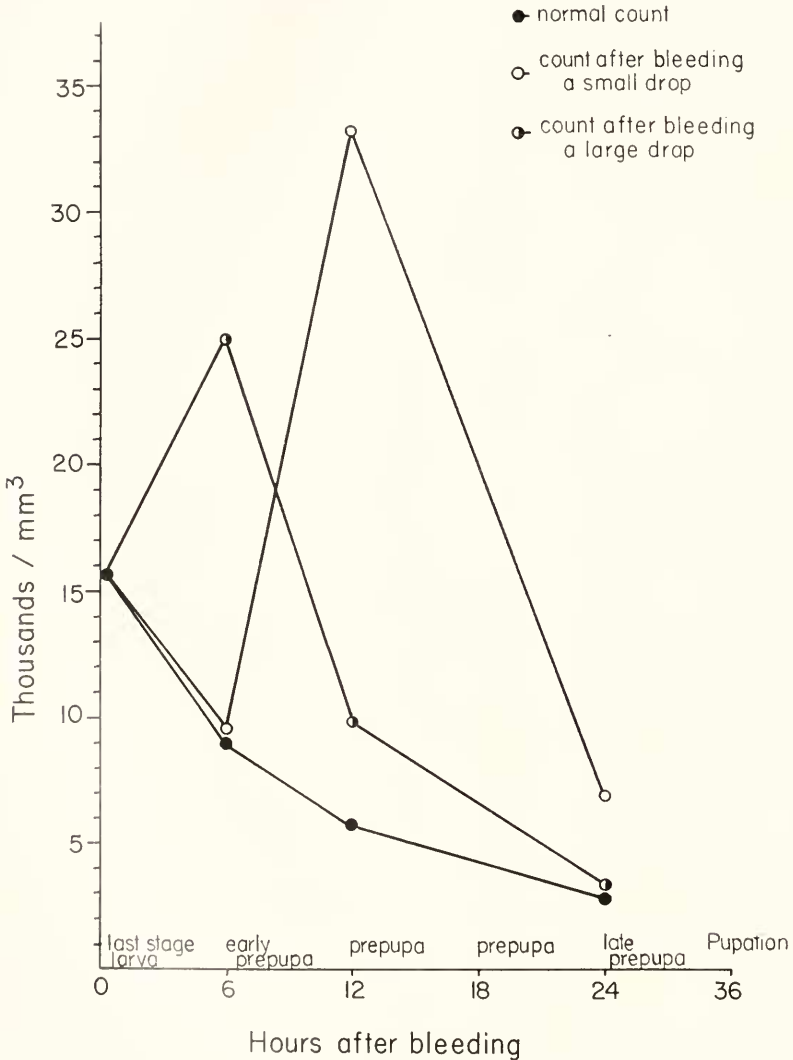

Figure 2. Curves showing the hemocyte concentration of larvae of *Galleria mellonella:* normal and after bleeding small and large drops.
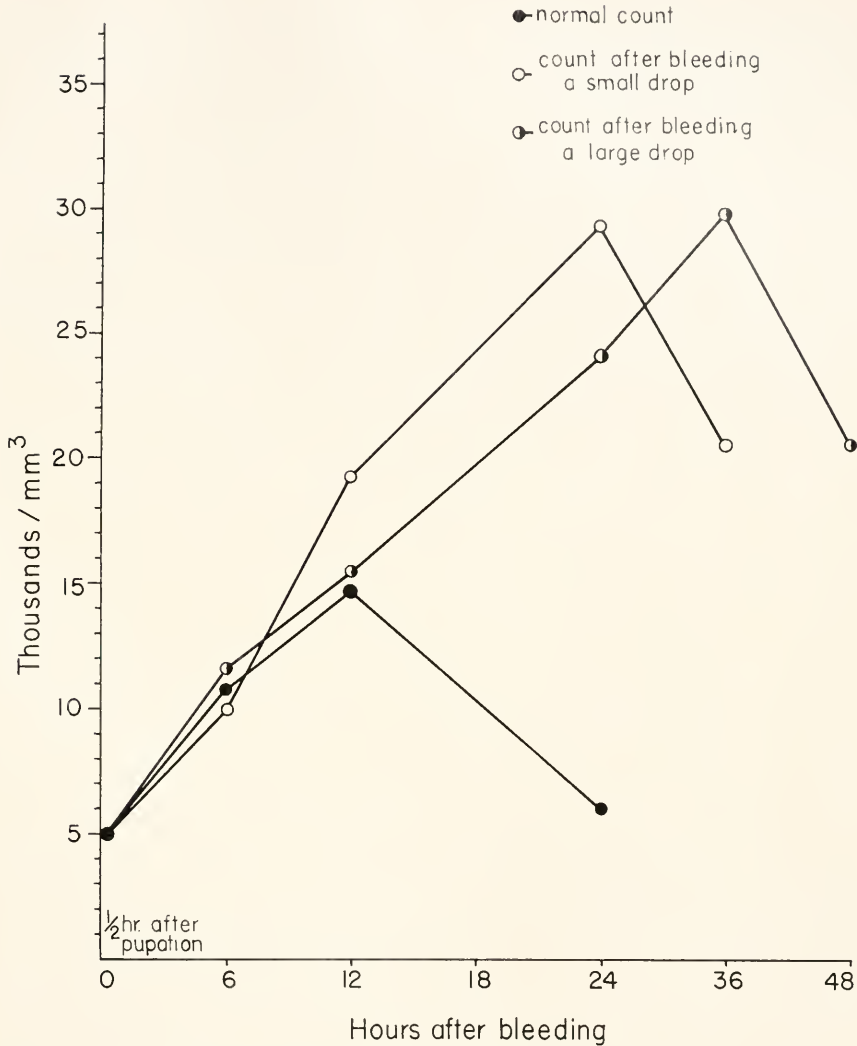
FIG. 3. Curves showing the hemocyte concentration of pupae of *Galleria mellonella*: normal and after bleeding small and large drops.

The conclusion from the above tests was to bleed larvae or pupae, then immediately inject H³ thymidine. After 6–24 hours, depending on the amount of bleeding and the age of the individuals, blood could then be used for transfusion or individuals joined in parabiosis.

The life cycle and life expectancy of hemocytes

Dakhnoff (1938) attempted to trace the transformation of regenerating blood cells (induced by bleeding) of *G. mellonella* by comparing differential HC counts taken every hour. He arrived at a transformation time of an hour or so. But,

as is evident, such curves could give only the speed of change in the differential hemocyte picture, not the actual time required for change. His method would not prove with certainty anything about the course of differentiation of individual hemocyte types.

To obtain definitive evidence, last instar larvae were bled a large drop (*ca.* 5 μl.) and then injected with 5 μl. of H³ thymidine. Blood smears were made at repeated intervals, different specimens being used for each interval to avoid any complications that might arise from repeated bleeding of individuals. The blood films obtained were fixed with ethanol, dried, and autoradiographed. The final slides, after development, were examined by phase contrast microscopy without staining.

The hemocyte picture varies greatly from one group of insects to another. In *G. mellonella* there are five distinct types of hemocytes: prohemocytes, plasmatocytes, adipohemocytes, spherule cells and oenocytoids (photomicrographs given in Ashhurst and Richards, 1964b). It is generally thought that prohemocytes differentiate into the other types, or at least into plasmatocytes which may later develop fat droplets to become adipohemocytes. In the present study the following data were obtained:

Time after injection of H³ thymidine	Picture of labeling
6 hours	*ca.* 10% of prohemocytes labeled
12 hours	some of labeled prohemocytes beginning to differentiate into plasmatocytes
24 hours	numerous plasmatocytes labeled, some beginning to develop fat droplets, few labeled prohemocytes
48 hours	both labeled plasmatocytes and labeled adipohemocytes present
72 hours	only adipohemocytes labeled
	(Pupation at this time)
96 hours	only adipohemocytes labeled
120 hours	only a few labeled adipohemocytes present
144 hours	no labeled hemocytes of any type

The above data are interpreted as showing that prohemocytes become labeled promptly, and that some of these soon differentiate into plasmatocytes which, with the development of numerous sudanophilic vacuoles, become adipohemocytes. In three days the sequence prohemocyte → plasmatocyte → adipohemocyte has been completed, and within six days the labeled adipohemocytes have vanished. It follows that the life expectancy of this cell type, at least at this stage in the life cycle of *G. mellonella,* is less than six days at 35° C.

The picture of blood cell differentiation is complicated by changes in hemocyte concentration treated in the preceding section and by changes in differential cell counts. All of the above cell types are found in late larval and early pupal life, but adipohemocytes become more numerous and more conspicuous after pupation. These changes, however, do not interfere with the use of labeled cells as presented in the next section.

Two other less common types of hemocytes are found in the blood of *G. mellonella.* These are the spherule cells and the oenocytoids. These two types
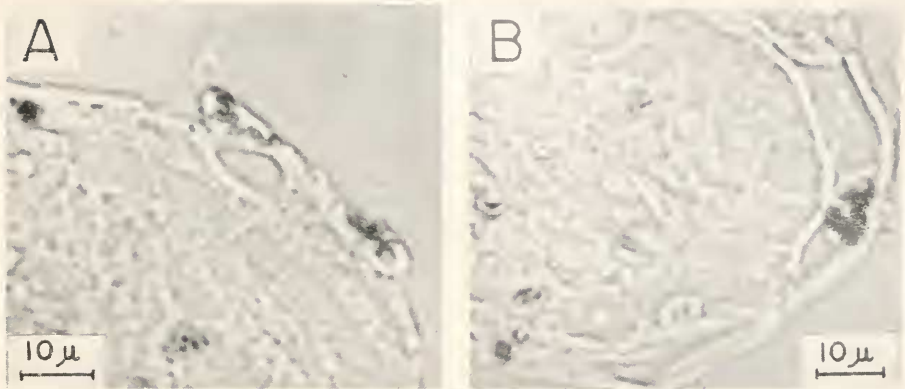
FIGURE 4. A. Autoradiograph showing labeled hemocytes in contact with the outer surface of the neural lamella of the nerve cord of *Galleria mellonella* 18 hours after pupation. B. Autoradiograph showing labeled hemocytes between laminae of the disintegrating neural lamella of the nerve cord of *Galleria mellonella* at about 18 hours after pupation.

are extremely difficult to identify with surety in our autoradiographed smears; hence nothing will be said about their origin or fate.

The relationship between hemocytes and connective tissue

The neural lamella around the central nervous system breaks down during the period 12–24 hours after pupation (Ashhurst and Richards, 1964a). Sections were prepared from 18-hour pupae which had received transfusions of labeled blood 6–48 hours previously. Labeled hemocytes were found throughout the hemocoel; some of these are appressed to the nerve cord prior to dissolution of
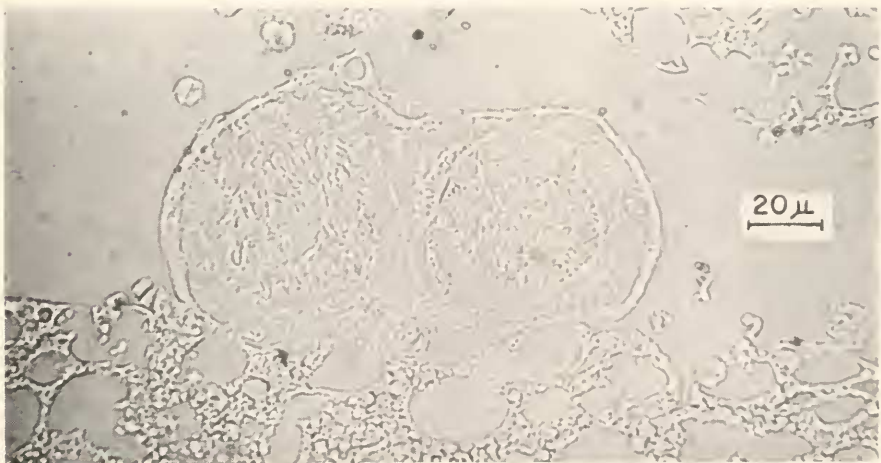

FIGURE 5. Autoradiograph of the abdominal nerve cord of *Galleria mellonella* 72 hours after pupation. This individual had been transfused with labeled blood. It shows the forming adult neural lamella without any label or labeled cells.

the neural lamella (Fig. 4 A), and, at a somewhat later stage, are found within the disintegrating laminae of the neural lamella (Fig. 4 B).

During the period 24–60 hours after pupation there is no neural lamella around the nerve cord (the central nervous system becomes reorganized to the adult condition during this time). A new neural lamella for the adult then begins to be formed and is clearly distinct by 72 hours (Ashhurst and Richards, 1964a). Sections prepared from 72-hour pupae which had been transfused with labeled blood or joined in parabiosis with a labeled pupa showed some labeled hemocytes in the hemocoel or appressed to various organs (especially fat body and tracheae) but no label in the sheath cells which are thought to secrete the neural lamella and no label in the neural lamella or immediately adjacent tissues (Fig. 5).

These data support the conclusion of Ashhurst and Richards that adipohemocytes have some intimate role in the destruction of the larval neural lamella during early pupal stages, but that neither these nor other hemocytes participate in the formation of the connective tissue around the adult nerve cord.

SUMMARY

1. The hemocyte count of *Galleria mellonella* during the prepupal and early pupal stages varies from 4000/mm.^{3} to 15000/m^3 as a function of age. The number of freely circulating cells can be approximately doubled within 6–24 hours by bleeding the insect.

2. Using tritiated thymidine and autoradiography it was found that there is a hemocyte differentiation sequence of prohemocyte → plasmatocyte → adipohemocyte. This differentiation requires about three days at 35°, and the labeled cells have disappeared in another three days. The life expectancy of these cells, at least in prepupal and early pupal stages, is then less than six days.

3. Transfusion of labeled hemocytes, followed by autoradiography of sections, confirmed the previous suggestion that adipohemocytes have some role in the destruction of the larval neural lamella, but that no blood cells are involved in the formation of the connective tissue around the adult nervous system.

LITERATURE CITED

Ashhurst, D. E., and A. G. Richards, 1964a. A study of the changes occurring in the connective tissue associated with the central nervous system during the pupal stage of the wax moth, *Galleria mellonella* L. *J. Morph.*, **114**: 225–236.

Ashhurst, D. E., and A. G. Richards, 1964b. Some histochemical observations on the blood cells of the wax moth, *Galleria mellonella* L. *J. Morph.*, **114**: 247–253.

Bonhag, P. F., and W. J. Arnold, 1961. Histology, histochemistry and tracheation of the ovariole sheaths in the American cockroach, *Periplaneta americana* L. *J. Morph.*, **108**: 107–129.

Dakhnoff, A., 1938. La regeneration du sang chez *Galleria mellonella* après une prise de sang. *C. R. Soc. Biol.*, **128**: 520–523.

Haydak, M. H., 1940. The length of development of the greater wax moth. *Science*, **91**: 525.

Joftes, D. L., 1959. Liquid emulsion autoradiography with tritium. *Lab. Investig.*, **8**: 131–136.

Jones, J. C., 1962. Current concepts concerning insect hemocytes. *Amer. Zool.*, **2**: 209–246.

Lazarenko, T., 1925. Beiträge zur vergleichenden Histologie des Blutes und des Bindegewebes. *Zeitschr. mikr. anat. Forsch.*, **3**: 409–499.

SCHARRER, B., 1939. The differentiation between neuroglia and connective tissue sheath in the cockroach (*Periplaneta americana*). *J. Comp. Neurol.*, **70**: 77–88.

SHRIVASTAVA, S. C., AND A. G. RICHARDS, 1964. The differentiation of blood cells in the wax moth, *Galleria mellonella*. *Amer. Zool.*, **4**: 312–313.

STEPHENS, J. M., 1963. Effect of active immunization on total hemocyte counts of larvae of *Galleria mellonella* L. *J. Ins. Path.*, **5**: 152–156.

WERMEL, E. M., 1938. Die Explantation des Blutes von Seidenspinnerraupen. *Bull. Biol. Med. Exp. URSS*, **5**: 6–9.

WHEELER, R. E., 1963. Studies on the total hemocyte count in *Periplaneta americana*. *J. Ins. Physiol.*, **9**: 223–236.

WIGGLESWORTH, V. B., 1956. The hemocytes and connective tissue formation in an insect, *Rhodnius prolixus* (Hemiptera). *Quart. J. Micr. Sci.*, **97**: 89–98.

WOODS, P. S., AND J. H. TAYLOR, 1959. Studies of ribonucleic acid metabolism with tritium labeled cytidine. *Lab. Investig.*, **8**: 309–318.